

## Hypothesis

A putative proton binding site of plasma membrane H<sup>+</sup>-ATPase identified through homology modellingJens T. Bukrinsky<sup>a,b</sup>, Morten J. Buch-Pedersen<sup>b</sup>, Sine Larsen<sup>a</sup>, Michael G. Palmgren<sup>b,\*</sup><sup>a</sup>Centre for Crystallographic Studies, Department of Chemistry, University of Copenhagen, Universitetsparken 5, DK-2100 Copenhagen, Denmark<sup>b</sup>Department of Plant Biology, The Royal Veterinary and Agricultural University, Thorvaldsensvej 40, DK-1871 Frederiksberg C, Denmark

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**Abstract** We have used the 2.6 Å structure of the rabbit sarcoplasmic reticulum Ca<sup>2+</sup>-ATPase isoform 1a, SERCA1a [Toyoshima, C., Nakasako, M., Nomura, H. and Ogawa, H. (2000) *Nature* 405, 647–655], to build models by homology modelling of two plasma membrane (PM) H<sup>+</sup>-ATPases, *Arabidopsis thaliana* AHA2 and *Saccharomyces cerevisiae* PMA1. We propose that in both yeast and plant PM H<sup>+</sup>-ATPases a strictly conserved aspartate in transmembrane segment (M)6 (D684<sub>AHA2</sub>/D730<sub>PMA1</sub>), and three backbone carbonyls in M4 (I282<sub>AHA2</sub>/I331<sub>PMA1</sub>, G283<sub>AHA2</sub>/I332<sub>PMA1</sub> and I285<sub>AHA2</sub>/V334<sub>PMA1</sub>) comprise a binding site for H<sub>3</sub>O<sup>+</sup>, suggesting a previously unknown mechanism for transport of protons. Comparison with the structure of the SERCA1a made it feasible to suggest a possible receptor region for the C-terminal auto-inhibitory domain extending from the phosphorylation and anchor domains into the transmembrane region. © 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

**Key words:** Auto-inhibition; H<sup>+</sup>-ATPase; Hydronium ion; Proton binding site; Regulatory domain; Rabbit sarcoplasmic reticulum Ca<sup>2+</sup>-ATPase isoform 1; Proton pump

## 1. Introduction

Plasma membrane (PM) H<sup>+</sup>-ATPases are responsible for the ATP-fueled ejection of protons out of plant and yeast cells [1,2]. An electrochemical gradient of protons across the PM is hereby generated comprising the driving force for solute uptake through other PM transport systems. PM H<sup>+</sup>-ATPases belong structurally and enzymatically to a large group of proteins termed P-type ATPases, also including the mammalian Na<sup>+</sup>/K<sup>+</sup>-, H<sup>+</sup>/K<sup>+</sup>-, PM Ca<sup>2+</sup>-, and sarcoplasmic reticulum

(SR) Ca<sup>2+</sup>-ATPases. A characteristic feature of P-type ATPases is that a strictly conserved aspartate residue is phosphorylated during the reaction cycle (reviewed in [3]).

P-type ATPases consist of a single catalytic subunit and appear to have a very similar fold. Crystallographic images to 8 Å resolution from the *Neurospora* fungal PM H<sup>+</sup>-ATPase reveal the presence of 10 transmembrane spanning helices (M1–M10) connected by a large cytoplasmic portion of the enzyme, and very little protein extending to the extracellular site of the cell [4]. In a comparison between this H<sup>+</sup> pump structure and an 8 Å resolution structure of the SERCA1a SR Ca<sup>2+</sup>-ATPase [5] it was concluded that these pumps share a great deal of structural similarity especially pronounced in the membrane region, despite a relatively low level of amino acid homology [6]. The X-ray crystal structure of the SERCA1a Ca<sup>2+</sup>-ATPase was recently determined to 2.6 Å resolution [7] and this structure has contributed with valuable knowledge and critical insights into the structure and mechanism of P-type ATPases. The cytoplasmic part of the protein is divided into three distinct parts: the nucleotide binding domain (N-domain), the phosphorylation domain (P-domain), and the actuator or anchor domain (A-domain) (Fig. 1). Many P-type ATPases, including PM H<sup>+</sup>-ATPases, in addition contain terminal auto-inhibitory regulatory domains (R-domains) at either the extreme N- or C-terminus. The structural basis for auto-inhibition of these pumps is unknown.

Here we report a model for a putative proton binding site in the transmembrane region of two PM H<sup>+</sup>-ATPases, AHA2 from *Arabidopsis thaliana* and PMA1 from *Saccharomyces cerevisiae*. The two models are produced by homology modelling based on the 2.6 Å resolution structure of SERCA1a.

## 2. Building the models of AHA2 and PMA1

The models were constructed using the coordinates from the structure of SERCA1a (Protein Data Base entry 1eul; [7]) with the programs Swiss-model and Swiss-pdb viewer [8,9]. Turbo-Frodo [10] was used as a graphical display program to visually validate the models and to adjust side chain conformations.

The major differences between the structure of SERCA1a (Fig. 1A) and the models of PMA1 (Fig. 1B) and AHA2 (Fig. 1C) are found in the N-domain, where large deletions in the sequences of PM H<sup>+</sup>-ATPases had to be accounted for. The final model of PMA1 comprises residues 15–400, 408–484, 489–516 and 521–918, while that of AHA2 comprises the res-

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**Abbreviations:** A-domain, actuator or anchor domain; ACA2, *Arabidopsis thaliana* auto-inhibited Ca<sup>2+</sup>-ATPase isoform 2; AHA2, *Arabidopsis thaliana* PM H<sup>+</sup>-ATPase isoform 2; M1–M10, transmembrane segments 1–10; N-domain, nucleotide binding domain; P-domain, phosphorylation domain; PM, plasma membrane; PMA1, *Saccharomyces cerevisiae* PM H<sup>+</sup>-ATPase isoform 1; PMA2, *Nicotiana plumbaginifolia* PM H<sup>+</sup>-ATPase isoform 2; R-domain, C-terminal regulatory domain; SERCA1a, rabbit SR Ca<sup>2+</sup>-ATPase isoform 1a; SR, sarcoplasmic reticulum

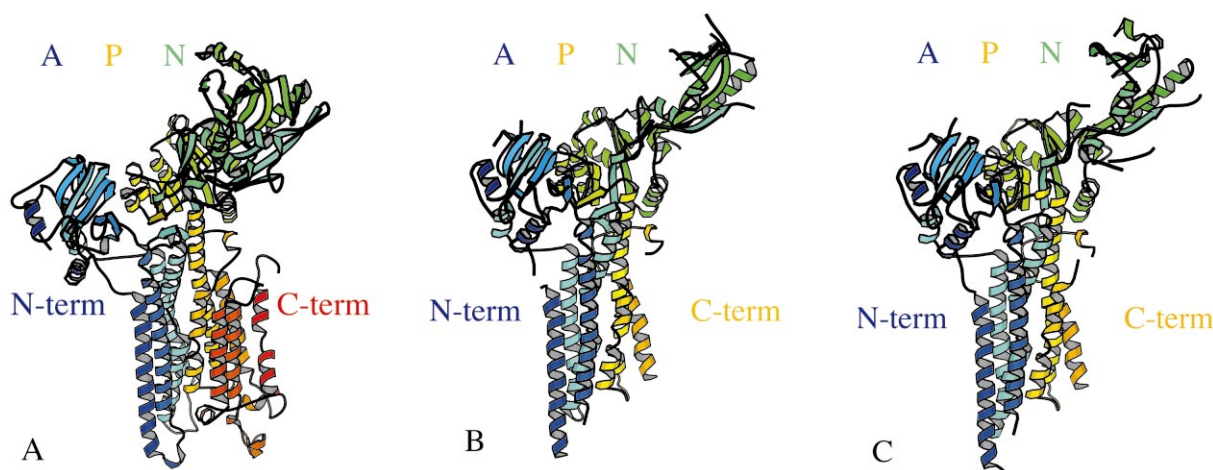


Fig. 1. Ribbon images of the X-ray structure of SERCA1a (A) and the most reliable part of the homology models of the PM  $H^+$ -ATPases AHA2 (B) and PMA1 (C). The structure of SERCA1a is according to Toyoshima et al. [7]. Ribbons are colored blue (N-terminus) through cyan, green, yellow, and orange to red (C-terminus). A, P and N denote A-, P- and N-domains, respectively. The figure was drawn using Mol-script [26].

idues 1–21, 31–87, 92–191, 195–347, 358–382, 389–400, 406–467, 474–691, 698–812 and 820–912. For the gaps in the model the remaining residues would allow the chains to be ligated without geometric strain. The C-terminal hydrophilic portion of AHA2 and PMA1 was not included due to lack of this domain in the SR  $Ca^{2+}$ -ATPases. The last four transmembrane helices display very low sequence homology between the  $Ca^{2+}$ - and  $H^+$ -ATPases and different prediction models lead to slightly contradictory results in the assignment of secondary structure. We have therefore not included this part in our presentation of the models (Fig. 1B,C). All shown side chains are modelled in their most common rotamer. The essential parts of the models with highly conserved patches such as the phosphorylation site and the nucleotide binding site appeared to be fully superimposable on the structure of SERCA1a  $Ca^{2+}$ -ATPase (data not shown), indicating a good overall agreement of the structures modelled.

### 3. Proton transport

#### 3.1. How does the PM $H^+$ -ATPase transport protons across the cell membrane?

The mechanism of proton pumping by PM  $H^+$ -ATPases is not known. It has been suggested that PM  $H^+$ -ATPases pump protons via a proton-wire mechanism (reviewed in [11]). However, in other P-type ATPases cations appear to be translocated by a mechanism involving specific binding site(s) in the membrane, with the accessibility alternating between the two sites of the membrane during the catalytic cycle. Due to the biochemical and structural similarity to other P-type ATPases, a similar transport mechanism seems likely for the PM  $H^+$ -ATPase [12]. Transport of protons via a mechanism involving alternating access to a specific proton binding site(s) would probably require the transported species to be  $H_3O^+$  rather than  $H^+$  [11,13]. Specific binding of a transported  $H_3O^+$  is likely to involve three or four liganding groups per bound  $H_3O^+$  as hydrogen-bonding partners.

#### 3.2. The region in $H^+$ -ATPases corresponding to site I of SERCA1a

In the 2.6 Å structure of the SR  $Ca^{2+}$  pump, two  $Ca^{2+}$  ions

are found in the transmembrane region [7] as seen in Fig. 2A. Site I (Fig. 2A,  $Ca^{2+}$  binding site located to the left) is located between M5 and M6, whereas site II (Fig. 2A,  $Ca^{2+}$  binding site located to the right) is located between M4 and M6. In site I,  $Ca^{2+}$  is interacting with three negatively charged residues E771<sub>SERCA1a</sub> (M5), D800<sub>SERCA1a</sub> (M6) and E908<sub>SERCA1a</sub> (M8). From sequence comparison of the transmembrane region of PM  $H^+$ -ATPases, it appears that only one negatively charged residue (D684<sub>AHA2</sub>/D730<sub>PMA1</sub>, corresponding to D800<sub>SERCA1a</sub>; M6) is strictly conserved. The sequence comparisons also suggest that the negatively charged residues E771<sub>SERCA1a</sub> and E908<sub>SERCA1a</sub> could be replaced by R655<sub>AHA2</sub>/H701<sub>PMA1</sub> (M5) and Q795<sub>AHA2</sub>/T837<sub>PMA1</sub> (M8) with the latter localized in a more dubious part of the models.

In the model of AHA2, the guanidinium group of R655<sub>AHA2</sub> occupies a position equivalent to that occupied by  $Ca^{2+}$  in site I of SERCA1a. The side chain of H701<sub>PMA1</sub> also protrudes into this region. Thus, according to our models, binding of  $H_3O^+$  seems very unlikely at this location in both PM  $H^+$ -ATPases. An arginine (R695<sub>PMA1</sub>) located six residues upstream from H701<sub>PMA1</sub> is conserved in the fungal PM  $H^+$ -ATPases (see the P-type ATPase database <http://www.biobase.dk/~axe/Patbase.html>). Based on genetic studies it was suggested that R695<sub>PMA1</sub> is linked by a salt bridge to D730<sub>PMA1</sub> in M6 [14]. The formation of this salt bridge is not confirmed by the homology model of PMA1, as it would require dramatic changes of modelled structure. It should be noted that significant distortions of the model are also necessary when an alternative sequence alignment of M5 [14] was used.

#### 3.3. The region in $H^+$ -ATPases corresponding to site II of SERCA1a

$Ca^{2+}$  binding site II of SERCA1a is formed by side chain oxygen atoms of E309<sub>SERCA1a</sub> (M4), N796<sub>SERCA1a</sub> (M6) and D800<sub>SERCA1a</sub> (M6) and three carbonyl oxygen atoms from M4 (those of V304<sub>SERCA1a</sub>, A305<sub>SERCA1a</sub> and I307<sub>SERCA1a</sub>) (Fig. 2A). In a perfect  $\alpha$ -helix all backbone carbonyl groups are involved in hydrogen-bonding with the backbone amide from the residue positioned +4 relative to the first. The carbonyl groups are exposed only if the helix is perturbed from

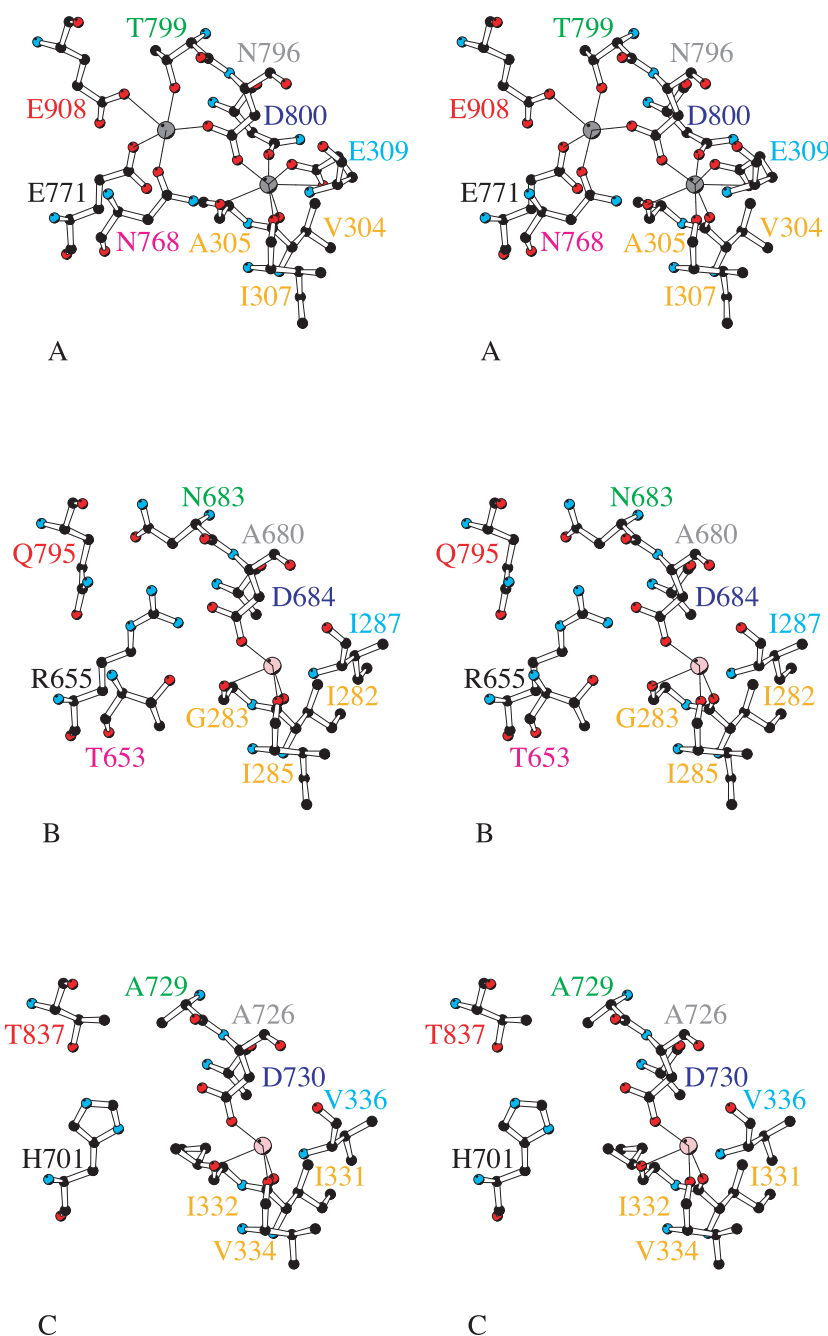


Fig. 2. Stereo view of the  $\text{Ca}^{2+}$  binding site of SERCA1a (A) and the proposed  $\text{H}_3\text{O}^+$  binding sites in the PM  $\text{H}^+$ -ATPases AHA2 (B) and PMA1 (C). All sites are shown with the same orientation. In the structure of SERCA1a [7], the  $\text{Ca}^{2+}$  ions are shown as gray spheres. The  $\text{Ca}^{2+}$  ions to the left and right are bound to site I and site II, respectively. In the PM  $\text{H}^+$ -ATPase models, a putative  $\text{H}_3\text{O}^+$  in site II is shown as a pink sphere. Residues with identical label colors are structurally aligned in the models. The figure was drawn using Molscrip [26]. Q795<sub>AHA2</sub>/T837<sub>PMA1</sub> are positioned in a less reliable part of the model.

ideal bonding geometry. Two proline residues, P308<sub>SERCA1a</sub> and P312<sub>SERCA1a</sub>, introduce unwinding of M4 by twisting of the backbone, in this way depriving the carbonyls of their natural hydrogen-bonding partners. The PM  $\text{H}^+$ -ATPases contain proline residues in equivalent positions which are strictly conserved (P286<sub>AHA2</sub>/P335<sub>PMA1</sub> and P290<sub>AHA2</sub>/P339<sub>PMA1</sub>) suggesting that M4 is also unwound in these pumps. In the homology models of AHA2 and PMA1, the backbone carbonyls of I282<sub>AHA2</sub>/I331<sub>PMA1</sub>, G283<sub>AHA2</sub>/I332<sub>PMA1</sub> and I285<sub>AHA2</sub>/V334<sub>PMA1</sub>, respectively, could contribute to a hydronium ion binding site.

### 3.4. The proposed $\text{H}^+$ binding site

The two homology models predict that one potential hydronium ion binding site is likely to be positioned in the region corresponding to  $\text{Ca}^{2+}$  binding site II. Three backbone carbonyls (those of I282<sub>AHA2</sub>/I331<sub>PMA1</sub>, G283<sub>AHA2</sub>/I332<sub>PMA1</sub> and I285<sub>AHA2</sub>/V334<sub>PMA1</sub>) comprise the hydrogen-bonding partners to  $\text{H}_3\text{O}^+$  and the carboxylic group of D684<sub>AHA2</sub>/D730<sub>PMA1</sub> could be sharing a proton with a bound hydronium ion and/or with R655<sub>AHA2</sub>/H701<sub>PMA1</sub>.

Experimental evidence points to a role for D684<sub>AHA2</sub> in the transport mechanism of AHA2. Thus, a D684N<sub>AHA2</sub> substi-

tution results in total abolishment of proton pumping [15]. The mutated enzyme is able to hydrolyze ATP but is blocked during its catalytic cycle immediately following formation of the  $E_1P$  phospho-intermediate. This suggests that the negatively charged side chain of D684<sub>AHA2</sub> interacts with the transported species and is essential for proton translocation although it may not be essential for binding to occur [15]. Efforts to express corresponding D730<sub>PMA1</sub> mutants were unsuccessful [14].

In SERCA1a five negatively charged residues, two polar residues and three backbone carbonyls form the binding sites for two  $Ca^{2+}$  ions. However, in PM  $H^+$ -ATPases only one negatively charged residue is strictly conserved (D684<sub>AHA2</sub>/D730<sub>PMA1</sub>). The region corresponding to  $Ca^{2+}$  binding site I in the homology models is unlikely to be able to bind  $H_3O^+$  due to the lack of excess negative charge. Furthermore, R655<sub>AHA2</sub>/H701<sub>PMA1</sub> is protruding into the region where  $Ca^{2+}$  is bound in SERCA1a. Excess of negative charge is found only in the region corresponding to  $Ca^{2+}$  site II and originates from the aspartate (D684<sub>AHA2</sub>/D730<sub>PMA1</sub>), the backbone carbonyls, and the helix dipole. In this otherwise hydrophobic environment a bound water molecule would tend to become protonated. This region in PM  $H^+$ -ATPases would thus have a relatively high affinity for one hydronium ion.

#### 4. Regulation and auto-inhibition of PM $H^+$ -ATPases

##### 4.1. In vivo and in vitro regulation of PM $H^+$ -ATPases

The activities of plant and yeast PM  $H^+$ -ATPases are down-regulated by an inhibitory domain located in the extreme C-terminus of the enzyme (R-domain). Deletion of the R-domain, either at the gene level [16,17] or by trypsin treatment [18,19], results in constitutively activated  $H^+$ -ATPases. The R-domain is likely to interact with a specific intramolecular receptor of the pump and in this way to exert its negative effect on enzyme activity. Based on peptide cross-linking studies, it has been proposed that the C-terminal R-domain of human PM  $Ca^{2+}$ -ATPase hPMCA4 interacts with the A-domain [20] and the N-domain [21] of this pump.

Using genetic approaches, a large number of amino acid residue substitutions leading to increased pump activity of *S. cerevisiae* (PMA1) [22] and *Nicotiana plumbaginifolia* (PMA2) [23,24] PM  $H^+$ -ATPases have been identified. These mutations, apart from those in the R-domain, are localized in the A-, N- and P-domains as well as in transmembrane regions. A similar strategy was used to identify regulatory mutants in *A. thaliana* (ACA2), a  $Ca^{2+}$  pump regulated by an N-terminal auto-inhibitory domain [25]. Such genetic information can be interpreted as evidence for domain interactions.

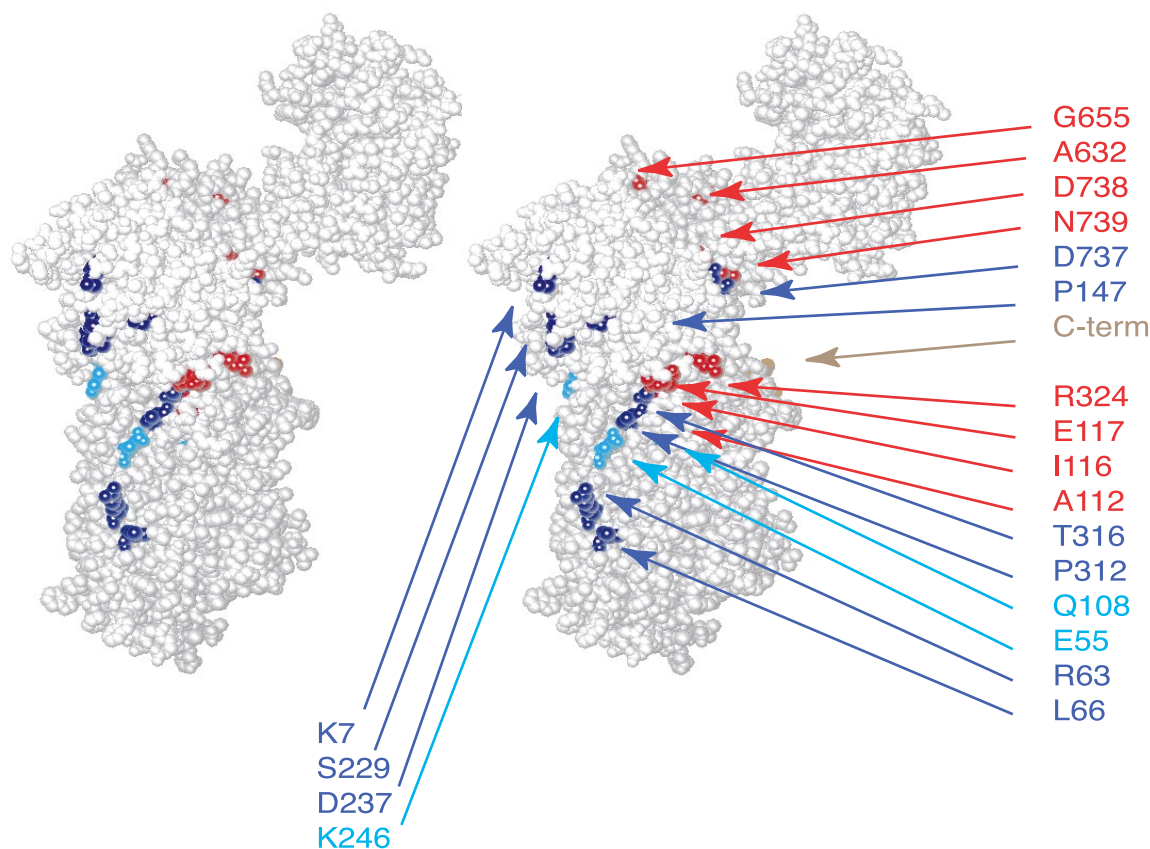


Fig. 3. Space-filling image in stereo view of the SERCA1a structure. Corresponding residues in *S. cerevisiae* PM  $H^+$ -ATPase PMA1 [22], *N. plumbaginifolia* PM  $H^+$ -ATPase PMA2 [23,24] and the *A. thaliana* endoplasmic reticulum  $Ca^{2+}$ -ATPase ACA2 [25] that give rise to activated mutants when substituted are indicated in red, blue, and green, respectively. The C-terminal is indicated in brown. Protein sequences were aligned using the Clustal W Multiple Sequence Alignment Program [27]. The indicated PMA1 residues correspond to the following SERCA1a residues: A165<sub>PMA1</sub>/A112<sub>SERCA1</sub>, (V169–D170<sub>PMA1</sub>)/(I116–E117)<sub>SERCA1</sub>, A351<sub>PMA1</sub>/R324<sub>SERCA1</sub>, A565<sub>PMA1</sub>/A632<sub>SERCA1</sub>, G587<sub>PMA1</sub>/G655<sub>SERCA1</sub>, P669<sub>PMA1</sub>/D738<sub>SERCA1</sub>, and G670<sub>PMA1</sub>/N739<sub>SERCA1</sub>. Residues in PMA2 correspond to residues in SERCA1a as follows: E14<sub>PMA2</sub>/K7<sub>SERCA1</sub>, P72<sub>PMA2</sub>/R63<sub>SERCA1</sub>, W75<sub>PMA2</sub>/L66<sub>SERCA1</sub>, P154<sub>PMA2</sub>/P147<sub>SERCA1</sub>, H221<sub>PMA2</sub>/S229<sub>SERCA1</sub>, H229<sub>PMA2</sub>/D237<sub>SERCA1</sub>, P294<sub>PMA2</sub>/P312<sub>SERCA1</sub>, S298<sub>PMA2</sub>/T316<sub>SERCA1</sub> and E626<sub>PMA2</sub>/D737<sub>SERCA1</sub>. Residues in ACA2 correspond to the following SERCA1a residues: E167<sub>ACA2</sub>/E55<sub>SERCA1</sub>, D219<sub>ACA2</sub>/Q108<sub>SERCA1a</sub> and E341<sub>ACA2</sub>/K246<sub>SERCA1</sub>. The figure was made with Turbo-Frodo [10].



However, because the mutations are scattered throughout the primary structure of the pumps, it has been difficult to build a model for the R-domain interactions.

#### 4.2. Structural basis of the regulation of PM $H^+$ -ATPases

The residues in SERCA1a that correspond to the residues in *S. cerevisiae* PMA1, *N. plumbaginifolia* PMA2, and *A. thaliana* ACA2 that lead to an activated enzyme when substituted are indicated in the space-filling image of SERCA1a shown in Fig. 3. Most of these residues cluster on the surface of the pump where they form an almost continuous line extending from the P-domain to the middle of M1 (Fig. 3). A similar pattern is revealed if the mutants are marked in the modelled structures of AHA2 and PMA1 (data not shown). This suggests that the C-terminus in the non-activated pump docks onto the ATPase molecule in a path running from the P-domain to the middle of the membrane in a plane almost perpendicular to the membrane. The C-terminal domain of plant PM  $H^+$ -ATPases is approximately 60 residues longer than that of fungal  $H^+$ -ATPases [19] and may therefore be able to extend further into the membrane than its yeast counterpart.

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